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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

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Improved method for the identification and characterization of interacting molecules using automation

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IMPROVED METHOD FOR THE IDENTIFICATION AND CHARACTERIZATION OF INTERACTING MOLECULES USING AUTOMATION

The present invention relates to an improved method for the identification and optionally the characterization of interacting molecules designed to detect positive clones from the rather large numbers of false positive clones isolated by conventional two-hybrid systems. The method of the invention relies on a novel combination of selection steps used to detect clones that express interacting molecules from false positive clones. The present invention further relates to a kit useful for carrying out the method of the invention. The present invention provides for parallel, high-throughput or automated interaction screens for the reliable identification of interacting molecules.

Protein-protein interactions are essential for nearly all biological processes like replication, transcription, secretion, signal transduction and metabolism. Classical methods for identifying such interactions like co-immunoprecipitation or cross-linking are not available for all proteins or may not be sufficiently sensitive. Said methods further have the disadvantage that only by a great deal of energy, potentially interacting partners and corresponding nucleic acid fragments or sequences may be identified. Usually, this is effected by protein sequencing or production of antibodies, followed by the screening of an expression-library.

An important development for the convenient identification of protein-protein interactions was the yeast two-hybrid (2H) system presented by Fields and Song (1989).This genetic procedure not only allows the rapid demonstration of in vivo interactions, but also the simple isolation of corresponding nucleic acid sequences encoding for the interacting partners. The yeast two-hybrid system makes use of the features of a wide variety of eukaryotic transcription factors which carry two separable functional domains: one DNA binding domain as well as a second domain which activates the RNA-polymerase complex (activation domain). In the classical 2H system a so-called "bait" protein comprising of a

DNA binding domain (GAL4bd or lex A) and a protein of interest „X" are expressed as a fusion protein in yeast. The same yeast cell also simultaneously expresses a so called "fish" protein comprising of an activation domain (GAL4ad or VP16) and a protein „Y". Upon the interaction of a bait protein with a fish protein, the DNA binding and activation domains of the fusion proteins are brought into close proximity and the resulting protein complex triggers the expression of the reporter genes, for example, HIS3 or lacZ. Said expression can be easily monitored by cultivation of the yeast cells on selective medium without histidine as well as upon the activation of the lacZ gene. The genetic sequence encoding, for example, an unknown fish protein, may easily be identified by isolating the corresponding plasmid and subsequent sequence analysis. Meanwhile, a number of variants of the 2H system have been developed. The most important of those are the "one hybrid" system for the identification of promoter binding proteins and the "tri-hybrid" system for the identification of RNA-protein-interactions (Li and Herskowitz, 1993; SenGupta et al., 1996; Putz et al., 1996).

The classical 2H system for the identification of protein-protein-interaction, has, until today, only been carried out on a laboratory scale. The various steps of this system need to be conducted serially. They are, therefore, quite time consuming. As a consequence, the 2H system has so far proven unsuitable for the analysis of eukaryotic library vs library screens to investigate protein-protein networks. Although recent developments have taken into account these disadvantages (Bartel et al.,1996), a successful large scale search of interacting proteins, for example on the basis of a eukaryotic library vs. library screen, has not been reported. More importantly, also all of the so far developed 2H systems suffer from the serious drawback that many false-positive clones not representing any interactions between binding partners are isolated. This is particularly inconvenient in cases where large numbers of clones are to be analyzed because in the case of a eukaryotic library vs library screen it is typical that several hundreds of thousands of clones have to be analyzed for the investigation of protein-protein networks. In particular, it is predicted that around 5 % of DNA binding ("Bait") fusion proteins may activate

the readout system without the need for any interacting fusion protein, and hence be classed here as false positives (Bartel et al., 1996). The isolation of such false positive clones is, in laboratory practice, rather troublesome. This is in particular true if a large number of clones is to be analysed.

The technical problem underlying the present invention was therefore to overcome these prior art difficulties and to furnish a system that reliably produces clones that express interacting molecules. This system should, moreover, be suitable for large-scale library vs library screens using a parallel, high-throughput or automated approach.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a method for the identification of at least one member of a pair or complex of interacting molecules, comprising:

(a) providing host cells containing at least two genetic elements with different selectable markers, said genetic elements each comprising genetic information specifying one of said members, at least one of said genetic elements that further specifies an activation domain fusion protein additionally comprising a counterselectable marker, said host cells further carrying a readout system that is activated upon the interaction of said molecules;

(b) allowing at least one interaction, if any, to occur;

(c) selecting for said interaction by transfering progeny of said host cells in a regular grid pattern effected by automation to:

(ca) at least one selective medium, wherein said selective medium allows growth of said host cells only in the absence of said counterselectable marker and in the presence of a selectable marker; and/or

(cb) a further selective medium that allows identification of said host cells only on activation of the readout system;

(d) identifying host cells that contain molecules that:

(da) do not activate said readout system on said at least one selective medium specified in (ca); and

(db) activate said readout system on said selective medium specified in (cb); and

(e) identifying at least one member of said pair or complex of interacting molecules.

In order to efficiently conduct a library vs. library screen, preferably a eukaryotic library vs library screen for interacting proteins, it was surprisingly found in accordance with the present invention that it is sufficient to identify only those proteins fused to a DNA binding domain which are able to activate the readout system without the need for any interacting fusion protein. Inclusion of an automation step as a feature of the method of the invention has a number of significant advantages as compared to prior art methods that we addressed in more detail herein below.

Preferably, said interaction is a specific interaction.

The terms "identification" and "identifying", as used in accordance with the present invention, relaté to the ability of the person skilled in the art to detect positive clones that express interacting molecules from false positive clones due to the activation of the readout system on the selective media and optionally additionally to characterize at least one of said interacting molecules by one or a set of unambiguous features. Preferably, said molecules are characterized by the DNA sequence encoding them, upon nucleic acid hybridization or isolation and sequencing of the respective DNA molecules. Alternatively and less preferred, said molecules may be characterized by different features such as molecular weight, isoelectric point and, in the case of proteins, the N-terminal amino acid sequence etc. Methods for determining such parameters are well known in the art.

Preferably, said members specified by said genetic elements are connected to a further entity that will upon the interaction activate or contribute to the

activation of said read out system. It is further preferred that said entity is conserved for each type of genetic element and that different types of genetic elements comprise different entities. It is additionally preferred that said member of said pair or complex of interacting molecules forms, when transcribed as RNA from said genetic element, an RNA transcript fused with RNA specifying said entity. Most preferably, said fused RNA transcript is translated to form a fusion protein comprising said member fused to said entity. As will be elaborated further herein below, said entity may be in one type of genetic element a DNA sequence encoding a DNA-binding domain and in a different type of genetic element a transactivating protein domain. Preferably, said genetic elements are vectors such as plasmids. The at least two genetic elements comprised in said host cell are preferentially vectors from a library such as a cDNA or genomic library. Thus, the method of the invention allows the screening of a variety of host cells wherein the vector portion of said genetic elements is preferably the same for each type of genetic element whereas the potentially interacting molecules are representatives of a library and, thus, as a rule and in case that the library has not been amplified, may differ in each host cell. In this connection the term "type of genetic element" refers to an element characterized by comprising the same entity, selectable and counterselectable markers.

Preferably, the "interaction" of said molecules is specific and characterized by a high binding constant. However, the term "interaction" may also refer to a binding between molecules with a lower binding constant which, however, must be sufficient to activate the readout system. The interaction that is detectable by the method of the invention preferably leads to the formation of a functional entity having a biological, physical or chemical activity which was not present in said host cell before said interaction occurred.

Said interaction may preferably lead to the formation of a functional transcriptional activator comprising a DNA-binding and a transactivating protein domain and which is capable of activating a responsive moiety that drives the activation of said readout system. For example, said moiety may be a promoter.

Alternatively, said interaction may lead to a detectable fluorescence resonance energy transfer obtained by the interaction of fusion proteins containing, for example, the GFP type a and GFP type b fluorescent proteins (Cubbitt et al., 1995).

In a further embodiment, said interaction may lead to a detectable modification of a substrate by an enzyme such as a color reaction obtained by the cleavage of a propeptide by an enzyme. In all these embodiments of the invention, it is understood that the interacting molecules are preferably directly fused to the molecules driving the readout system.

The term "growth" on selective media "in the absence of at least one of said counter-selectable markers" refers to the fact that a population of host cells containing at least a pair of genetic elements is placed on said selective media but only those progeny of the host cells in the overall population that have lost the relevant genetic element are able to grow. For example, when a yeast strain which is resistant to the drug cycloheximide (cyh2) and which also contains a plasmid carrying the wild-type CYH2 gene (Kaeufer et al., 1983)) is placed on a selective medium containing cycloheximide, only those progeny of the yeast strain that have lost the plasmid carrying the CYH2 gene are able to grow, because this gene confers sensitivity to cycloheximide in yeast cells.

With reference to step (ca), it should be noted that the at least one selective medium would comprise at least one counterselectable compound such as cycloheximide; it would further typically lack a compound complementing for an auxotrophic marker or comprise an antibiotic. The compound or antibiotic may be the same for the various selective media.

The method of the present invention provides a highly effective tool for selecting against false positive clones that have proven to dramatically reduce the overall usefulness of the two-hybrid system. For example, by inclusion of a marker counterselecting for the absence of a genetic element that specifies the activation domain fusion protein, clones that will grow and therefore only carry

the genetic element specifying the DNA binding domain fusion protein can now be tested for the activation of the readout system. If this clone containing only the DNA binding domain fusion protein activates the readout system in the absence of the genetic element that encodes the activation domain fusion protein, then it will be classified as a false positive. Thus, only clones that activate the readout system in the presence of both genetic elements, but do not activate the read out system when the genetic element encoding the activation domain fusion protein is lost are classified as positives.

The advantages associated with the method of the invention have a significant impact in particular on the number of clones that express potentially interacting partners that can conveniently be analyzed. For example, even work on the laboratory scale will be more effective since positive clones that express interacting partners can be easily and unambiguously discriminated from false positive clones without the generation of additional strains. In contrast, to detect false positive clones using the state of the art yeast two-hybrid system, plasmids that encode bait proteins usually need to be isolated and retransformed into yeast cells harboring no other plasmids or harboring plasmids that encode unrelated fish proteins. Further, the enormous number of false positive clones that would be isolated when using the classical two-hybrid system on a large scale, yet are discriminated by the method of this invention no longer precludes an effective high through-put analysis of clones. In the long run, it is expected that the method of the present invention is especially advantageous for a high throughput analysis of a large number of yeast clones containing interacting molecules since many specific interactions and the individual members of these interactions can be identified in a parallel and automated approach.

Some investigators have noted the problem of identifying false positive clones when applying the yeast two-hybrid system in the past. Bartel et al. (1996) described a method for the elimination of false positives by replica plating clones that express one fusion protein from SD-leu and SD-trp plates, to SD-his plates. Clones that showed growth on the SD-his plates where identified as

false positives and were subsequently not used for interaction mating. The disadvantage of this method is that the procedure is labor intensive because yeast strains expressing the fish proteins, the bait proteins and the potentially interacting fish and bait proteins all must be generated and analyzed. Secondly, Bartel and coworkers used a GAL4 UAS to control the readout system, which is more likely to be bound by activation domain fusion proteins to generate a false positive signal than the bacterial LexA UAS used in one preferred embodiment of the method of the invention. The use of the counterselectable system described in this invention has the advantage that only one strain which expresses the potentially interacting fusion proteins is generated and must be analyzed.

A schematic overview of one embodiment of the method of the invention is provided in Figure 1. For the parallel analysis of a network of protein-protein interactions with the method of the invention, a library of plasmid constructs that express DNA binding domain and activation domain fusion proteins is provided. These libraries may consist of specific DNA fragments or a multitude of unknown DNA fragments ligated into the improved binding domain and activating domain plasmids of the invention containing different selectable and counterselectable markers. Both libraries are combined within yeast cells by transformation or interaction mating, and yeast strains that express potentially interacting proteins are selected on selective medium lacking histidine. The selective markers TRP1 and LEU2 maintain the plasmids in yeast strains grown on selective media, whereas CAN1 and CYH2 specify the counter-selectable markers that select for the loss of each plasmid. HIS3 and lacZ represent selectable markers integrated into the yeast genome, which are expressed on activation by interacting fusion proteins.

The readout system is, in the present case, both growth on medium lacking histidine and enzymatic activity of ß-galactosidase which can be subsequently screened. It is to be understood, however, that the readout system may rely on only one marker such as HIS3. Yet, the combination of two components that constitute the readout system in many cases allows a more ready interpretation

of results, in particular if one of the components, when activated, effects a change in color. A colony picking robot is used to pick the resulting yeast colonies into individual wells of 384-well microtiter plates containing selective medium lacking histidine, and the resulting plates are incubated at 30°C to allow cell growth. The interaction library contained in microtiter plates can be optionally replicated and stored. The resulting interaction library is investigated to detect positive clones that express interacting proteins and discriminate them from false positive clones using the method of the invention. Using a spotting robot, cells are transferred to replica membranes which are subsequently placed onto the selective media SD-leu-trp-his and SD-trp+CHX. After incubation on the selective plates, the clones grown on the membranes are subjected to a ß-Gal assay and a digital image from each membrane is obtained with a CCD camera which is then stored on computer. Using digital image processing and analysis (Lehrach et al. 1997) clones that express interacting fusion proteins can be identified by considering the pattern of ß-Gal activity from clones grown on the various selective media. The individual members comprising interactions can then be identified by one or more techniques, including PCR, sequencing, hybridization, oligofingerprinting or antibody reactions. An actual experiment carried out along the schematic route presented in Figure 1 is shown in Figures 4, 5, 6, 7, and 8.

The genetic elements specified here and above may further and advantageously be equipped with at least two different selection markers functional in bacteria such as E.coli. Such selection markers, for example aphA (Pansegrau et al., 1987) or bla allow the easy separation of said genetic elements upon retransformation into E.coli strains.

In a preferred embodiment of the method of the present invention said pair or complex of interacting molecules is selected from the group consisting of RNA-RNA, RNA-DNA, RNA-protein, DNA-DNA, DNA-protein, protein-protein, protein-peptide, or peptide-peptide interactions.

Accordingly, the method of the invention is applicable in a wide range of biological interactions. For example, the invention will be useful in identifying peptide-protein or peptide-peptide-interactions by employing synthetic peptide libraries (Yang et al., 1995).

Two applications of interests are the application of a large scale two-hybrid system for the detection of protein-protein interactions involved in medically relevant pathways which may be useful as therapeutic targets for the treatment of disease, and a large scale tri-hybrid system which is one example of said complex of interacting molecules mentioned herein above for the identification of, for example, novel post-transciptional regulators and their binding sites (SenGupta et al., 1996; Putz et al., 1996). In this regard it should be noted that a complex, in accordance with the invention may comprise more than three interacting molecules. Furthermore, such a complex may be composed of biologically or chemically different members. For example, to identify interacting RNA binding proteins and RNA molecules, a plasmid expressing a LexA-HIV-1Rev protein, a plasmid transcribing an RNA sequence in fusion with the Rev responsive element and a plasmid expressing a potentially RNA-interacting protein in fusion with an activation domain may be present in one cell. The plasmids encoding the RNA fusion molecule and the activation domain fusion protein must contain different selectable and counterselectable markers according to the method of the invention. If the RNA fusion molecule interacts with the respective two fusion proteins, the readout system is activated. To test whether the RNA fusion molecule or the activation domain fusion protein interact, the method of the invention is used to investigate the activation of the readout system in the absence of either of these fusion molecules.

In a further preferred embodiment, said genetic elements are plasmids, artificial chromosomes, viruses or other extrachromosomal elements.

Whereas it is preferred, due to the easy handling, to employ plasmids that specify the genetic elements in accordance with the present invention, the persons skilled in the art will be able to devise other systems that carry said genetic elements and that are identified above.

In an additional preferred embodiment, said readout system is a detectable protein. A number of readout systems are known in the art and may, if necessary, be adapted to be useful in the method of the invention.

Most preferably, said detectable protein is that encoded by the gene lacZ, HIS3, URA3, LYS2, sacB or HPRT, respectively. As is well known in the art, the expression of the ß-gal enzyme in yeast can be used for the formation of a detectable blue colony after incubation in X-Gal solution. Of course, the method of the invention is not restricted for use of only one readout system. On the contrary, if desired, a number of such readout systems may be combined. Said combination of a number of readout systems is, in accordance with the present invention, also comprised by the term "readout system". Such a combination will provide an additional safe guard for the identification of clones containing interacting partners.

Although the two-hybrid system has been developed in yeast, the method of the invention can be carried out in a variety of host systems. Preferred of those are yeast cells, bacterial cells, mammalian cells (Wu et al. 1996), insect cells or plant cells. Preferably, the bacterial cells are E. coli cells.
Of course, the genetic elements may be engineered and prepared in one host organism and then, e.g. by employing shuttle vectors, be transferred to a different host organism where it is employed in the method of the invention.

In another preferred embodiment, the method of the present invention comprises transforming or transfecting said host cell with at least one of said genetic elements prior to step (a).
Whereas the person skilled in the art may initiate the identification method of the invention starting from fully transformed or transfected host cells, he may wish to first generate such host cells in accordance with the aim of his research or commercial interest. For example, he may wish to generate a certain type of library first that he intends to screen against a second library already present in said host cells. Alternatively, he may have in mind to generate two different

libraries that he wants to screen against each other. In this case, he would need to first transform said host cells, simultaneously or successively, with both types of genetic elements.

In another preferred embodiment, said host cells with said genetic elements are generated by cell fusion, conjugation or interaction mating.

The biological principal of counter-selection referred to above is well known in the art. Accordingly, the person skilled in the art may chose from a variety of such counter-selectable markers. Preferably, said markers are CAN1, CYH2, LYS2, URA3, HRPT or sacB.

It is further preferred in accordance with the present invention that said selectable markers are auxotrophic or antibiotic markers.

It is important to note that some of the markers that are used as a readout system, may also be used as selectable markers. It is further important to note that one and the same marker can not be used as selectable marker and as part of the readout system at the same time.

Most preferably, said auxotrophic or antibiotic markers are selected from LEU2, TRP1, URA3, HIS3, ADE2, LYS2 and Zeocin.

Planning of experiments may require that the test for interaction need not be done immediately after the provision of host cells and, possibly, the occurrence of the interactions. In such cases, the researcher may wish to store the transformed host cells for further use. Accordingly, a further preferred embodiment of the invention relates to a method wherein progeny of host cells obtained in step (b) are transferred to a storage compartment.

In particular in cases where a large number of clones is to be analyzed, said transfer is advantageously effected or assisted by automation or a picking robot. How such a picking robot may actually be put into practice, is described

for example in Lehrach et al. (1997). Naturally, other automation or robot systems that reliably pick progeny of said host cells into predetermined arrays in the storage compartments may also be employed.

The host cells will, in this embodiment, be propagated in said storage compartment and provide further progeny for the additional tests. Preferably, replicas of said storage compartment maintaining the array of clones are set up. Said storage compartments comprising the transformed host cells and the appropriate media may be maintained in accordance with conventional cultivation protocols. Alternatively, said storage compartments may comprise an anti-freeze agent and therefore be appropriate for storage in a deep-freezer. This embodiment is particularly useful when the evaluation of potential interacting partners is to be postponed. As is well known in the art, frozen host cells may easily be recovered upon thawing and further tested in accordance with the invention. Most preferably, said anti-freeze agent is glycerol which is preferably present in said media in an amount of 3 - 25% (vol/vol).

In a further particularly preferred embodiment of the method of the invention, said storage compartment is a microtiter plate. Most preferably, said microtiter plate comprises 384 wells. Microtiter plates have the particular advantage of providing a pre-fixed array that allows the easy replicating of clones and furthermore the unambiguous identification and assignment of clones throughout the various steps of the experiment. The 384 well microtiter plate is, due to its comparatively small size and large number of compartments, particularly suitable for experiments where large numbers of clones need to be screened.

Depending on the design of the experiment, the host cells may be grown in the storage compartment such as the above microtiter plate to logarithmic or stationary phase. Growth conditions may be established by the person skilled in the art according to conventional procedures. Cell growth is usually performed between 15 and 45 degrees Celsius.

Transfer of said host cells in step (c) made or assisted by automation is made by using a spotting robot or by using a pipetting or micropipetting device. How such a spotting robot may be devised and equipped is, for example, described in Lehrach et al. (1997). Naturally, other automation or robctic systems that reliably create ordered arrays of clones may also be employed.

Most advantageously, said transfer is effected in a regular grid pattern at densities of 1 to 1000 clones per square centimeter.

Most preferably, said transfer is made to a planar carrier which is subsequently placed on the at least two selective media as specified in steps (ca) and (cb). Alternatively, said transfer of said host cells may be made to the planar carrier already placed on the selective media or said transfer may be made directly to the selective media.

In order to increase the population of host cells available for growth on said selective medium in (ca) it is most advantageous to make multiple transfers that carry additional host cells of the same yeast strain to the same position in said regular grid. Preferably, the number of said multiple transfers is between two and 20 times. If said múltiple transfer is made or assisted by a spotting robot it is most advantageous for each transfer to be made from a slightly different position of the microtitre plate well containing said yeast strain.

The progeny of said host cells may be transferred to a variety of planar carriers. Most preferred is a membrane which may, for example, be manufactured from nylon, nitro-cellulose or PVDF.

The selective media used for growth of appropriate clones may be in liquid or in solid form. Preferably, said selective media when used in conjunction with a spotting robot and membranes as planar carriers are solidified with agar on which said spotted membranes are subsequently placed. Alternatively, and also preferably, said selective media when in liquid form are held within microtiter plates and said transfer is made by replication.

Referring now to the step (d) of the method of the invention, the readout system can be analyzed by a variety of means. For example, it can be analyzed by visual inspection, radioactive, chemiluminescent, fluorescent, photometric, spectrometric, infra red, colourimetric or resonant detection.

Preferably, said identification of host cells that express interacting fusion proteins is effected by visual means from consideration of the activation state of said readout system of clones grown on the at least two selective media as specified in steps (ca) and (cb).

Also preferably, said identification of host cells that express interacting fusion proteins in step (d) is effected or assisted by digital image storage, analysis or processing. In this embodiment, positive clones which are preferably arrayed on a planar carrier such as a membrane are identified by comparison of digital images obtained from the membrane after activation of said readout system on said selective media specified in (ca) and (cb).

Most preferably, the identity of positive host cells and false positive host cells are stored on computer, for example within a relational database.

Identification of the at least one member of the pair or complex of interacting molecules may be effected by a variety of means. For example, molecules can be characterized by nucleic acid hybridization, oligonucleotide hybridization, nucleic acid or protein sequencing, restriction digestion, spectrometry or antibody reaction. Once the first member of an interaction has been identified, the second member or further members can also be identified by any of the above methods. Preferably the identification of at least one member of an interaction is effected by nucleic acid hybridization, antibody binding or nucleic acid sequencing.

If nucleic acid hybridization is to be carried out, the nucleic acid molecules comprised in the host cell and encoding for at least one of the interacting

molecules is preferably affixed to a planar carrier. As is well known in the art, said planar carrier to which said nucleic acid may be affixed, can be for example, a Nylon-, nitrocellusose- or PVDF membrane, glass or silica substrates (DeRisi et al. 1996; Lockhart et al. 1996). Said host cells containing said nucleic acid may be transferred to said planar carrier and subsequently lysed on the carrier and the nucleic acid released by said lysis is affixed to the same position by appropriate treatment. Alternatively, progeny of the host cells may be lysed in a storage compartment and the crude or purified nucleic acid obtained is then transferred and subsequently affixed to said planar carrier. Advantageously, said nucleic acids are amplified by PCR prior to transfer to the planar carrier. Most preferably said nucleic acid is affixed in a regular grid pattern in parallel with additional nucleic acids representing different genetic elements encoding interacting molecules. As is well known in the art, such regular grid patterns may be at densities of between 1 and 50 000 elements per square centimeter and can be made by a variety of methods. Preferably, said regular patterns are constructed using automation or a spotting robot such as described in Lehrach et al. (1997) and Maier et al. (1997) and furnished with defined spotting patterns, barcode reading and data recording abilities. Thus it is possible to correctly and unambiguously return to stored host cells containing said nucleic acid from a given spotted position on the planar carrier. Also preferably, said regular grid patterns may be made by pipetting systems, or by microarraying technologies as described by Shalon et al. (1996), Schober et al (1993) or Lockart et al. (1996). Identification is, again, advantageously effected by nucleic acid hybridization.

Using a detectable nucleic acid probe of interest, homologous nucleic acids which are affixed on the planar carrier can be identified by hybridization. From the spotted position of said homologous identified nucleic acid on the planar carrier, the corresponding host cell in the storage compartment can be identified which contains both or all members of the interaction. The for example second member of the interaction can now be identified by any of the above methods. For example, by use of a radioactively labeled Ras probe, homologous nucleic acids on the planar carrier can be identified by

hybridization. The Ras interacting proteins can now be identified from the corresponding host cell that contains both the first genetic element homologous to the Ras probe and the second genetic element encoding for these Ras interacting proteins.

If multiple oligonucleotide hybridizations are carried out on the nucleic acids affixed to the planar carrier, oligofingerprints of all genetic elements encoding the interacting proteins can be obtained. These oligofingerprints can be used to identify all members of the interactions or those members that belong to specific gene families, as described in Maier et al. (1997).

Advantageously, the nucleic acid molecules that encode the interacting proteins are, prior to identification such as by DNA sequencing, amplified by PCR or in said genetic elements in host cells and preferable in *E. coli*. Amplification of said genetic elements is conducted by multiplication of the *E. coli* cells and isolation of said genetic elements. Methods of identifying the nucleic acids that encode interacting proteins by DNA sequencing and analysis are well known in the art. By amplifying and sequencing the nucleic acids that encode for both or all members of an interaction from the same clone, the identity of both or all members of the interaction can be determined.

If a specific antibody is to be used to determine whether a protein of interest is expressed as a fusion protein within an interaction library, it is advantageous to affix all fusion proteins expressed from the interaction library on to a planar carrier. For example, clones of the interaction library that express fusion proteins can be transferred to a planar carrier using a spotting robot as described in Lehrach et al (1997). The clones are subsequently lysed on the carrier and released proteins are affixed onto the same position. Using, for example, an anti-HIP1-antibody (Wanker et al. 1997), clones from the interaction library that contain HIP1 fusion proteins and an unknown interacting fusion protein can be identified. The unknown member of the interacting pair of molecules can now be identified from the corresponding host cell by any of the above methods. The antibodies used as probes may be directly detectably

labeled. Alternatively, said antibodies may be detected by a secondary probe or antibody which may be specific for the primary antibody. Various alternative embodiments using, for example, tertiary antibodies may be devised by the person skilled in the art on the basis of his common knowledge.

Most advantageously, when said identification of members comprising an interaction is effected using said regular grids, a digital image of the planar carrier after hybridization or antibody reaction is obtained and analysis is effected by digital image storage, processing or analysis using an automated or semi-automated image analysis system, such as described in Lehrach et al. (1997).

Most preferably, the information comprising the identity of the host cell and the identity of the interacting molecules expressed by the genetic elements contained within the host cell are stored on a computer, for example within a relational database.

These data are available for the establishment of a network of interactions. By collecting the information from a whole interaction library, the inter-relationship between many different interacting molecules can be determined and thus enable the establishment of a network of interactions. Preferably, said data can be accessed though the use of software tools or graphical interfaces that enable the investigator to easily query the established interaction network with a biological question or to develop the established network by the addition of further data.

Advantageously, those molecules identified as interacting with many different molecules can be recorded. This information can reduce the work needed to further characterize particular interactions since those interactions comprising of a molecule found to interact with many other molecules within the yeast two-hybrid system may be suspected of being artifactual (Bartel et al., 1993).

A significant advantage of the method of invention over existing yeast two-hybrid systems is the scale at which such identification of interactions and interacting members can be made. Preferably, the method of the invention screens library vs library interactions using arrayed interaction libraries. Thus, the method of invention allows, in an efficient manner, a more complete and exhaustive generation of protein-protein interaction networks than existing methods. An established and exhaustive network of protein-protein interactions is of use for many purposes as shown Figure 2. For example, it may be used to predict the existence of new biological interactions or pathways, or to determine links between biological networks. Furthermore with this method, the function and localization of previously unknown proteins can be predicted by determining their interaction partners. It also can be used to predict the response of a cell to changes in the expression of particular members of the networks. Finally, these data can be used to identify proteins or interactions between proteins within a medically relevant pathway which are suitable for therapeutic intervention, diagnosis or the treatment of a disease.

In accordance with the present invention, it is additionally preferred prior to step (a) that a preselection against clones that express a single molecule able to activate the readout system is carried out on culture media comprising a counterselective compound, for example 5-fluoro orotic acid, canavanine, cycloheximide or α-amino-adipate .

In this embodiment, for example, the URA3 gene is incorporated as a component of the readout system. Clones containing only one of said genetic elements are placed on a selective medium comprising 5-fluoro orotic acid (5-FOA). In the case that clones that express a single molecule able to activate the readout system, 5-FOA is converted into the toxic 5-fluorouracil. Accordingly, host cells containing auto-activating molecules will die on the selective medium containing 5-FOA.

It is further important to note that the marker used for said preselection cannot be used as a selectable or counterselectable marker at the same time.

The present invention also relates to a method for the production of a pharmaceutical composition comprising formulation said at least one member of the interacting molecules identified by the method of the invention in a pharmaceutically acceptable form.

Said pharmaceutical composition comprises at least one of the aforementioned compounds isolated by the method of the invention, either alone or in combination, and optionally a pharmaceutically acceptable carrier or exipient. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by conventional methods. These pharmaceutical compositions can be administered to subject in need thereof at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{22} copies of the nucleic acid molecule. Proteins or peptides may be administered in the range of 0,1ng to 10mg per kg of body weight. The compositions of the invention may be administered locally or systematically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

The present invention further relates to a method for the production of a pharmaceutical composition comprising formulating an inhibitor of the

interaction of the interacting molecules identified by the method of the invention in a pharmaceutically acceptable form.

The inhibitor may be identified according to conventional protocols. Additionally, molecules that inhibit existing protein-protein interactions can be isolated with the yeast two-hybrid system using the URA3 readout system. Yeast cells that express interacting GAL4ad and LexA fusion proteins which activate the URA3 readout system are unable to grow on selective medium containing 5-FOA. However, when an additional molecule is present in these cells which disrupts the interaction of the fusion proteins the URA3 readout system is not activated and the yeast cells can grow on selective medium containing 5-FOA. Using this method potential inhibitors of a protein-protein interaction can be isolated from a library comprising these inhibitors. Systems corresponding to the URA3 system may be devised by the person skilled in the art on the basis of the teachings of the present invention and are also comprised thereby.

Also, the present invention relates to a method for the production of a pharmaceutical composition comprising identifying a further molecule in a cascade of interacting molecules, of which the at least one member of interacting molecules identified by any of the above methods is a part of or identifying an inhibitor of said further molecule.

Once at least one member of the interacting molecules has been identified, it is reasonable to expect that said member is a part of a biological cascade. Identification of additional members of said cascade can be effected either by applying the method of the present invention or by applying conventional methods. Also, inhibitors of said further members can be identified and can be formulated into pharmaceutical compositions.

The present invention relates further to a kit comprising at least one of the following:

(i) host cells as identified in any of the preceding claims and at least one genetic element comprising said genetic information

specifying at least one of said possibly interacting molecules and optionally containing a counter-selectable marker as specified herein above;

(ii) host cells as identified in any of the preceding claims and at least one genetic not comprising genetic information specifying at least one of said potential interacting molecules and optionally containing a counter-selectable marker as specified herein above;

(iii) at least one genetic element comprising said genetic information specifying at least one of said potentially interacting molecules and optionally containing a counter-selectable marker as specified herein above;

(iv) at least one genetic element not comprising genetic information specifying at least one of said potentially interacting molecules and optionally containing a counter-seletable marker as specified herein above;

(v) host cells comprising at least one and preferably at least two of said genetic elements specified in (iii) or (iv);

(vi) at least one planar carrier carrying nucleic acid or protein from said host cells comprising at least one member of said genetic elements specified herein above wherein said nucleic acid or protein is affixed to said carrier in grid form and optionally solutions to effect hybridization or binding of nucleic acid probes or proteins to said molecules affixed to said grid;

(vii) at least one storage compartment, planar carrier or computer disc comprising or/and characterizing genetic elements, host cells, storage compartments or carriers identified in any of (i) to (vi); and/or

(viii) at least one yeast strain comprising a *can*1 and a *cyh*2 mutation.

Preferably, said kit comprises or also comprises at least one storage compartment containing the host cells of (i), (ii) or (v) and/or comprises or also comprises at least one storage compartment containing said genetic

information or said potentially interacting molecules encoded by said genetic information as specified in (i) or (iii).

The present invention also relates to the use of any of the yeast strains described herein above and in the appended examples for the the identification of at least one member of a pair of potentially interacting molecules.

The figures show:

Figure 1 A schematic overview of one embodiment of the method of the invention.

For the parallel analysis of a network of protein-protein interactions using the method of the invention, a library of plasmid constructs that express DNA binding domain and activation domain fusion proteins is provided. These libraries may consist of specific DNA fragments or a multitude of unknown DNA fragments ligated into the improved binding domain and activating domain plasmids of the invention which contain different selectable and counterselèctable markers. Both libraries are combined within yeast cells by transformation or interaction mating, and yeast strains that express potentially interacting proteins are selected on selective medium lacking histidine. The selective markers TRP1 and LEU2 maintain the plasmids in the yeast strains grown on selective media, whereas CYH2 specifies the counter-selectable marker that selects for the loss of the activation domain plasmid. HIS3 and lacZ represent selectable markers in the yeast genome, which are expressed upon activation by interacting fusion proteins. The readout system is, in the present case, both growth on medium lacking histidine and the enzymatic activity of ß-galactosidase which can be subsequently screened. A colony picking robot is used to pick the resulting yeast colonies into individual wells of 384-well microtiter plates containing selective

medium lacking histidine, and the resulting plates are incubated at 30°C to allow cell growth. The interaction library held in the microtiter plates optionally may be replicated and stored. The interaction library is investigated to detect positive clones that express interacting fusion proteins and discriminate them from false positive clones using the method of the invention. Using a spotting robot, cells are transferred to replica membranes which are subsequently placed onto the selective media SD-leu-trp-his and SD-trp+CHX. After incubation on the selective plates, the clones which have grown on the membranes are subjected to a ß-Gal assay and a digital image from each membrane is obtained with a CCD camera which is then stored on computer. Using digital image processing and analysis (Lehrach et al. 1997) clones that express interacting fusion proteins can be identified by considering the pattern of ß-Gal activity of these clones grown on the various selective media. The individual members comprising the interactions can then be identified by one or more techniques, including PCR, sequencing, hybridization, oligofingerprinting or antibody reactions.

Figure 2 The applications of an established and exhaustive network of protein-protein interactions. The identity of positive clones and the identity of the members comprising the interactions for the entire interaction library can be stored in a database. These data are used to establish a network of protein-protein interactions which can be used for a variety of purposes. For example, they may be used to predict the existence of new biological interactions or pathways, or to determine links between biological networks. Furthermore with this method, the function and localization of previously unknown proteins can be predicted by determining their interaction partners. It also can be used to predict the response of a cell to changes in the expression of particular members of the networks. Finally, these data can be used to identify proteins

within a medically relevant pathway which are suitable for therapeutic, diagnosis intervention and for the treatment of disease.

Figure 3 Plasmids constructed for the improved 2-hybrid system.

a) the plasmid maps of pGAD428a, b and c activation domain vector series. The plasmids contain the unique restriction enzyme sites for *Sal* I and *Not* I which can be used to clone a genetic fragment into the multiple cloning site. The plasmids are maintained in yeast cells by the selectable marker LEU2. The loss of the plasmids can be selected for by the counterselective marker CYH2.

b) Polylinkers used within the multiple cloning site to provide expression of the genetic fragment in one of the three reading frames.

Figure 4 Predicted interactions between fusion proteins used to create the defined interaction library. The fusion proteins enclosed with dark rounded boxes are believed to interact as shown. The LexA-HIP1 fusion protein enclosed by a thin rectangular box has been shown to activate the LacZ readout system without the need for any interacting fusion protein. The two proteins LexA and GAL4ad, and the two fusion proteins GAL4ad-14-3-3 and LexA-MJD (all unboxed) are believed not to interact with each other or other fusion proteins used in this example.

Figure 5 Digital images of the ß-gal assays made from the replica Nylon membranes containing the spotted interaction library obtained from the selective media (a) SD-leu-trp-his and (b) SD-trp+CHX.
In each case, The left hand side of each membrane contains control clones and clones from the defined interaction library, and the right hand side contains only clones from the defined interaction library. The two regions marked on the first membrane represent those clones magnified in Figure 6. The overall size of each

membrane is 22 x 8 cm and contains 6912 spot locations at a spotting pitch of 1.4 mm.

Figure 6 Magnification of clones from the interaction library taken from the same region of three membranes obtained from the selective media SD-leu-trp-his and SD-trp+CHX assayed for β-gal activity:

A. Clones imaged from a region of the right hand side of the membrane containing the defined interaction library. Clones from the defined interaction library that express interacting proteins are ringed and correspond to the microtiter plate addresses 06L22 and 08N24.

B. Clones imaged from a region of the left hand side of the same membranes containing control clones and clones from the interaction library, where clones around each ink guide-spot are arranged as shown and correspond to: 00 Ink guide spot; 01False positive control clone that expresses the fusion protein GAL4ad-LexA; 02 False positive clone expressing the fusion protein LexA-HIP1; 03 Positive control clone expressing the interacting fusion proteins LexA-SIM1 & GAL4ad-ARNT; 04 Clone from the defined interaction library. The positive control clone (spot position 03) is ringed.

Figure 7 Identification by hybridization of the genetic fragments carried by the clones 06L22 and 08N24. A 1.3 kb, SIM1 and a 1.4 kb ARNT DNA fragment were used as nucleic acid probes for hybridization to high-density spotted membranes containing DNA from the defined interaction library. These clones were identified containing SIM1 and ARNT genetic fragments by hybridization. The images are of the same region of the membranes as those shown in Figure 6a. The spot positions of the clones 06L22 and 08N24 are ringed.

Figure 8 Identification of the SIM1 and ARNT DNA fragments from the yeast two hybrid plasmid carried by the clone 06L22 by duplex PCR. Plasmid DNA was isolated from a liquid culture of the clone 06L22 by a QiaPrep (Hilden) procedure and the inserts contained within the plasmids were amplified by PCR using the primer pairs, 5'-TCG TAG ATC TTC GTC AGC AG-3' & 5'-GGA ATT AGC TTG GCT GCA GC-3' for the plasmid pBTM117c and 5'-CGA TGA TGA AGA TAC CCC AC-3' & 5'-GCA CAG TTG AAG TGA ACT TGC-3' for pGAD426. Lane 1 contains a Lamda DNA digestion with *Bst*EII as size marker; Lane 2 contains the duplex PCR reaction from plasmids isolated from clone 06L22; Lanes 3 and contain control PCR amplifications from the plasmids pBTM117c-SIM1 and pGAD426-ARNT respectively.

The examples illustrates the invention.

Example 1. Construction of vectors and a novel host strain for an improved yeast two-hybrid system

The plasmids constructed for an improved yeast two-hybrid system pGAD428 a, b and c are shown in Fig. 3a. This set of vectors can be used for the construction of activation domain fusion proteins. The vectors contain the unique restriction sites *Sal* I and *Not* I located in the multiple cloning site (MCS) region at the 3´- end of the open reading frame for the GAL4ad sequence (Fig. 3b).

With this set of plasmids, activation domain fusion proteins are expressed at high levels in yeast host cells from the constitutive *ADH1* promoter (P) and the transcription is terminated at the *ADH1* transcription termination signal (T). The two-hybrid plasmids shown in Fig. 3a are shuttle vectors that replicate autonomously in both *E. coli* and *S. cerevisiae*.

The plasmids pGAD428 a, b and c are used to generate fusion proteins that contain the GAL4 activation domain (amino acids 768-881) operatively linked to a protein of interest. The plasmids pGAD428 a, b and c carry the wild type yeast *CYH2* gene, which confers sensitivity to cycloheximide in transformed cells (Kaeufer et al., 1983), the selectable marker *LEU2*, that allows yeast leu2- auxotrophs to grow on selective synthetic medium without leucine, and the bacterial marker *aphA* (Pansegrau et al., 1987) which confers kanamycin resistance in *E. coli*. The plasmids pGAD428a, b and c were created from pGAD427 by ligation of the adapters shown in Table 1 into the MCS to construct the improved vectors with three different reading frames.

For the construction of pGAD427 a 1.2 kb *Dde* I fragment containing the *aphA* gene was isolated from pFG101u (Pansegrau et al., 1987) and was subcloned into the *Pvu* I site of the pGAD426 using the oligonucleotide adapters 5'-GTCGCGATC-3' and 5'-TAAGATCGCGACAT-3'. The plasmid pGAD426 was generated by insertion of a 1.2 kb *Eco* RV *CYH2* gene fragment, which was isolated from the pAS2-1 (Clonetech) into the *Pvu* II site of pGAD425 (Han and Collicelli, 1995).

Table 1: Oligonucleotide adapters used for the construction of the novel yeast two-hybrid vectors pGAD428 a, b and c.

oligonucleotide	sequence (5'-3')
a sense	TCGAGTCGACGCGGCCGCTAA
a antisense	GGCCTTAGCGGCCGCGTCGAC
b sense	TCGAGGTCGACGCGGCCGCAGTAA
b antisense	GGCCTTACTGCGGCCGCGTCGACC
c sense	TCGAGAGTCGACGCGGCCGCTTAA
c antisense	GGCCTTAAGCGGCCGCGTCGACTC

To allow for the CHX counterselection provided by the improved two-hybrid vectors, the *S. cerevisiae* strain L40cc was created. L40cc is isogenic with strain L40c (Wanker *et al*, 1997.), except for the presence of a cyh2 mutation. This mutation was selected by plating L40c cells onto YPD plates containing 10 μg/ml cycloheximide (Sigma St Louis). The genotype of the L40cc strain is: *Mata his3Δ200 trp1-910 leu2-3,112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ Gal4 can1 cyh2.*

To determine whether the two-hybrid plasmids can be used for the detection of clones expressing interacting molecules from false positive clones several DNA fragments encoding proteins of interest were cloned into the vectors. The orientation of the inserted fragments was determined by restriction analysis and the reading frame was checked by sequencing. The generated constructs and the original plasmids described above are listed in Table 2. The construction of pBTM117c-HD1.6, -HD3.6 and -SIM1 was described elsewhere (Wanker et al., 1997; Probst et al., 1997). pBTM117c-HIP1 and pGAD426-HIP1 were obtained by ligation of a 1.2 kb *Sal* I HIP1 fragment isolated from pGAD-HIP1 (Wanker et al., 1997) into pBTM117c and pGAD426, respectively. pBTM117c-MJD was created by inserting a 1.1 kb *Sal* I/*Not* I MJD1 fragment (Kawagushi et al., 1994) into pBTM117c, and pGAD426-14-3-3 was generated by inserting a 1.0 kb EcoRI/NotI fragment of pGAD10-14-3-3 into pGAD426. For the construction of pGAD426-HIPCT, a 0.5 kb *Eco* RI HIP1 fragment isolated from pGAD10-HIPCT was ligated into pGAD426. pLEXA-HIP1 and pGAD426-ARNT were generated by inserting a 2.5 kb *Sph* I LexA-HIP1 fragment and a 1.4 kb *Sal* I/*Not* I ARNT fragment into pGAD426, respectively.

It was shown that the fusion proteins LexA-SIM1 and GAL4ad-ARNT specifically interact with each other in the yeast two-hybrid system (Probst et al., 1997), because when both hybrids were coexpressed in *Saccharomyces cerevisiae* containing two integrated reporter constructs, the yeast HIS3 gene and the bacterial lacZ gene, which both contained binding sites for the LexA protein in the promoter region, the interaction between these two fusion proteins led to the transcription of the reporter genes. The fusion proteins by

themselves were not able to activate transcription because GAL4ad-ARNT lacks a DNA binding domain and LexA-SIM1 an activation domain (Probst et al., 1997). In contrast it was shown recently that the fusion protein LexA-HIP1 is capable of activating the HIS3 and lacZ reporter genes without interacting with a specific GAL4ad fusion protein. Thus, the yeast clones expressing the LexA-HIP1 protein have to be designated as false positives, because false positives are defined here as clones where a LexA fusion protein alone without the respective partner protein activates the transcription of the reporter genes without the need for any interacting partner protein. To differentiate between positive clones that express interacting fusion proteins and false positives, an improved version of the two hybrid system described in this invention was developed.

Table 2: Two-hybrid vectors used for the expression of fusion proteins.

plasmid	fusionprotein	insert (kb)	counterselection	selection in yeast	reference
pBTM117c	lexA	-	CAN1	TRP1	Wanker et al. 1997
pBTM117c-HD1.6	lexA-HD1.6	1.6	CAN1	TRP1	Wanker et al., 1997
pBTM117c-HD3.6	lexA-HD3.6	3.6	CAN1	TRP1	Wanker et al., 1997
pBTM117c-SIM1	lexA-SIM1	1.1	CAN1	TRP1	Probst et al., 1997
pBTM117c-MJD	lexA-MJD	1.4	CAN1	TRP1	this work
pBTM117c-HIP1	lexA-HIP1	1.2	CAN1	TRP1	this work
pGAD426	GAL4ad	-	CYH2	LEU2	this work
pGAD426-ARNT	GAL4ad-ARNT	1.3	CYH2	LEU2	Probst et al., 1997
pGAD426-HIP1	GAL4ad-HIP1	1.2	CYH2	LEU2	Wanker et al., 1997
pGAD426-HIPCT	GAL4ad-HIPCT	0.8	CYH2	LEU2	Wanker et al., 1997
pGAD426-14-3-3	GAL4ad-14-3-3	1.0	CYH2	LEU2	this work
pLEXA-HIP1	lexA-HIP1	1.2	CYH2	LEU2	this work

Example 2. Detection and identification of interacting proteins using a large-scale and automated application of the improved 2-hybrid system.

A scheme utilizing the method of the invention within a large-scale and automated approach for the parallel detection of clones that express interacting fusion proteins and the identification of members comprising the interactions is shown in Figure 1. Yeast clones from an 'interaction library' that express interacting proteins are identified on a large-scale by the use of visual inspection or digital image processing and analysis of high-density spotted membranes on which their ß-galactosidase activity has been assayed after growth on various selective media. Automated methods based on those described in Lehrach *et al.* (1997) are used to effect the production of the interaction library and high-density spotted membranes, and the analysis of digital images of the ß-gal assay and hybridization images.

To prove that the method of the invention as described in Figure 1 could successfully identify positive clones that expressed interacting proteins from false positive clones, and then subsequently identify the individual members comprising the interaction, an experiment was conducted using well defined plasmid constructs for the expression of known fusion proteins. Some of these fusion proteins are known to interact with each other while others do not interact with any other fusion proteins in the defined system. The essential steps of the method shown in Figure 1 were used, and the results show that the method of the invention can be used as a high-throughput, parallel and automated approach to generate large amounts of data leading to the establishment of protein-protein interacting networks.

Generation of a well defined interaction library

To generate the well defined interaction library, a series of plasmid constructs were used. Table 2 lists the constructs used for the expression of the LexA or GAL4ad fusion proteins. The predicted protein-protein interactions of these

fusion proteins are shown in Figure 4. It was shown that the fusion proteins LexA-SIM1 & GAL4ad-ARNT and LexA-HD1.6 & GAL4ad-HIP1 specifically interact with each other in the yeast two-hybrid system because they only activate the reporter genes HIS3 and LacZ when both proteins are present in one cell (Probst et al. 1997; Wanker et al. 1997). In contrast, it was demonstrated that the LexA-HIP1 fusion protein is capable of activating the reporter genes without the need for any interacting fusion protein. The proteins LexA and GAL4ad and the fusion proteins LexA-MJD and GAL4ad-14-3-3 which are also present in the defined interaction library are unable to activate the reporter genes either alone or when present in the same cell with any other fusion proteins comprising the library.

To generate the well defined interaction library, the constructs for the expression of the nine fusion proteins shown in Figure 4 were pooled and 3 µg of the mixture was co-transformed into yeast strain L40cc by the method of Schiestel & Gietz (1989). The resulting transformants were plated onto large 24 x 24 cm agar plates (Genetix, UK) containing minimal medium lacking tryptophan, leucine and histidine (SD-leu-trp-his). After growth at 30°C for 4 days, individual yeast colonies were picked using a picking robot based on that described in Lehrach *et al.* (1997). With this robot, individual yeast colonies were picked into individual wells of a 384-well microtiter plates (Genetix, UK) containing SD-leu-trp-his/7% glycerol liquid medium. The resulting microtiter plates were incubated at 30°C for 3 days. Although yeast colonies are more difficult than *E. coli* cells to handle in automated systems, a picking success of approximately 80% was achieved. After growth of yeast strains within the microtiter plates, each plate was labeled with an individual number and barcode. Each plate was also replicated to create two additional copies using a sterile 384-pin plastic replicator (Genetix, UK) to transfer a small amount of cell material from each well into pre-labeled 384-well microtiter plates and pre-filled with SD-leu-trp-his/7% glycerol liquid medium. The replicated plates were incubated at 30 °C for 3 days, subsequently frozen and stored at -70 °C together with the original picked microtiter plates of the interaction library.

Generation of high-density spotted membranes for use in an improved yeast 2-hybrid approach

A high-throughput spotting robot such as that described by Lehrach *et al.* (1997) was used to construct filters with a high-density pattern of yeast clones from the defined interaction library contained within 384-well microtiter plates. The position of individual clones on the high-density filter was recorded by the robot by the use of a pre-defined duplicate spotting pattern and the barcode of the microtiter plate. Labeled membranes (Hybond N+, Amersham UK) were pre-soaked in SD-leu-trp-his medium and placed in the robot. The interaction library was automatically arrayed as replica copies onto the membranes using a 384-pin spotting tool affixed to the robot. Five different microtiter plates from the first copy of the interaction library were replica spotted in a '3x3 duplicate' pattern around a central ink guide-spot onto 10 nylon membranes - corresponding to approximately 1900 clones spotted at a density of approximately 35 spots per cm^2. On each replica membrane three different control clones were spotted, each from a microtiter plate that contained the same control clone in every well. One control clone expressed the fusion proteins LexA-SIM1 & GAL4ad-ARNT, a second control clone the fusion protein LexA-HIP1, while a third expressed fusion protein GAL4ad-LexA, and all were spotted in order to test the selection, counterselection and the ß-gal assay features of the method. To ensure the number of yeast cells on each spot was sufficient for those membranes which were to be placed on the counterselection media plates, the robot was programmed to spot onto each spot position 5 times from a slightly different position within the wells of the microtiter plates. The robot created a data-file in which the spotting pattern produced and the barcode that had been automatically read from each microtiter plate was recorded.

Each membrane was carefully laid onto approximately 300 ml of solid agar media in 24 x 24 cm assay trays. Six membranes were transferred to SD-leu-trp-his media and two of the remaining membranes were transferred to SD-trp+CHX medium. The yeast colonies were allowed to grow on the surface of the membrane by incubation at 30 °C for 3 days.

Detection of the readout system

Two membranes from each of the selective media were assayed for lacZ expression using the ß-gal assay as described by Breeden & Nasmyth (1985) and air dried overnight. For each membrane, a 32-bit digital image of the ß-gal assay was obtained with a high-resolution charge coupled device (CCD) color camera (Kontron, Germany), and the images were stored on computer. One image of the defined interaction library that was grown on membranes placed on each of the two selective media and subsequently assayed for β-gal activity is shown in Figure 5. Individual clones of the interaction library can be identified and their position on the high-density spotted filter converted to specific wells in the microtiter plates using a semi-automated screening system as described by Lehrach *et al.* (1997).

Positive clones that express interacting fusion proteins can be detected from false positive clones by considering the activity of ß-galactosidase of clones grown on spotted membranes laid on the selective media. Positive clones should activate the lacZ reporter gene on SD-leu-trp-his media and turn blue on incubation with X-Gal solution, but not on the counterselective medium SD-trp+CHX. False positive clones should activate the reporter gene and turn blue on incubation with X-Gal solution on the counterselective medium as well as on the SD-leu-trp-his medium.

Figure 6 shows magnified images of a ß-gal assay of clones grown on the membranes which had been placed on the two selective media. Within the magnified region of the membranes shown in Figure 6a, two clones were detected as positive clones that express interacting fusion proteins since they activated the lacZ reporter gene on SD-leu-trp-his media, but not on the counterselective medium, and whose spotted positions are circled. The two clones were identified by their microtiter plate address within the interaction library as 06L22 and 08N24 respectively. All other clones spotted within this region of the membrane were detected as false positive since they express ß-galactosidase on SD-trp+CHX medium as well as on SD-leu-trp-his medium.

Expression of the LacZ reporter gene for the three control clones spotted onto the same membranes confirm these results. The positive control clone that expresses the interacting fusion proteins LexA-SIM1 & GAL4ad-ARNT should show a LacZ+ phenotype when grown on SD-leu-trp-his medium, but LacZ- when grown on the counterselective medium SD-trp+CHX. This control clone was spotted at position 03 in the region of the membranes shown in Figure 6b, of which one example is circled. The pattern of ß-gal activity for this positive control clone on the two selective media is as predicted. The false positive control clone that expresses the fusion protein LexA-HIP1 is spotted at position 02. This false positive control clone shows a LacZ+ phenotype when grown on SD-leu-trp-his media, but is detected as a false positive clone by the method of the invention since it also shows a LacZ+ phenotype on the SD-trp+CHX medium.

Identification of individual members of the interaction

The interaction library constructed for this example was composed of known fusion proteins with predicted interactions as shown in Figure 4. A real positive clone from this defined interaction library is therefore expected to express the interacting fusion protein-pairs LexA-SIM1 & GAL4ad-ARNT or LexA-HD1.6 & GAL4ad-HIP1 and hence contain the corresponding pairs of plasmid constructs pBTM117c-SIM1 & pGAD426-ARNT or pBTM117c-HD1.6 & pGAD426-HIP1, respectively. The identification of individual members that comprise an interaction between fusion proteins that are expressed within a single cell can be made by a variety of means as outlined in Figures 1. and 2. Two independent methods, nucleic acid hybridization and PCR, were used to identify the individual plasmid constructs that expressed the interacting fusion proteins in the positive clones 06L22 and 08N24.

The four membranes which had been placed on the SD-leu-trp-his medium and had not been used to assay ß-gal activity were processed according to the procedure described in Larin & Lehrach (1990) in order to affix the DNA contained within the clones of the interaction library onto the surface of the

membrane. A 1.3 kb DNA fragment of SIM1 and a 1.4 kb DNA fragment of ARNT were radioactively labeled by standard random priming procedures for use as a hybridization probe (Feinberg & Vogelstein, 1983). Each probe was heat denatured for 10 min at 95 °C and hybridized overnight at 65 °C in 15 ml of 5% SDS/0.5M sodium phosphate (pH 7.2)/1 mM EDTA with a high-density spotted membrane with DNA from the interaction library affixed to it. The membranes were washed once in 40mM sodium phosphate/0.1%SDS for 20 min at room temperature and once for 20 min at 65 °C before wrapping each membrane in Saran wrap and exposing it overnight to a phosphor-storage screens (Molecular Dynamics, USA). A digital image of each hybridized membrane was obtained by scanning the phosphor-storage screen using a phosphor-imager (Molecular Dynamics, USA). The digital image was stored on computer and was analyzed using a semi-automated system as described in Lehrach *et al.* (1997) which marked positive hybridization signals with square blocks. Figure 7 shows a magnified region of each hybridized membrane corresponding to that shown in Figure 6a containing the clones 06L22 and 08N24, the spotting position of which are circled. These clones were predicted to express either the interacting fusion protein-pairs LexA-SIM1 & GAL4adARNT or LexA-HD1.6 & GAL4ad-HIP1, and hybridization with the specific SIM1 and ARNT probes have shown that both clones contain the plasmid constructs pBTN117c-SIM1 and pGAD426-ARNT.

The individual clone 06L22 was recovered from the frozen plates of the original interaction library and inoculated into SD-leu-trp-his liquid medium. This culture was allowed to grow for 3 days at 30 °C and the corresponding plasmids contained in the clone were isolated using a QiaPrep (Qiagen, Hilden) procedure. Duplex PCR was used to simultaneously amplify the inserts contained within the plasmid constructs using primer-pairs specific for either the pBTM117c or GAD426 plasmids. The presence of the SIM1 and ARNT inserts was confirmed for clone 06L22 by electrophoresis of the amplified PCR products against separate control amplifications of the inserts from plasmids pBTM117c-SIM1 and pGAD426-ARNT as size markers (Figure 8).

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CLAIMS

1. A method for the identification of at least one member of a pair or complex of interacting molecules, comprising:

(a) providing host cells containing at least two genetic elements with different selectable markers, said genetic elements each comprising genetic information specifying one of said members, at least one of said genetic elements that further specifies an activation domain fusion protein additionally comprising a counterselectable marker, said host cells further carrying a readout system that is activated upon the interaction of said molecules;

(b) allowing at least one interaction, if any, to occur;

(c) selecting for said interaction by transfering progeny of said host cells in a regular grid pattern effected by automation to:

(ca) at least one selective medium, wherein said selective medium allows growth of said host cells only in the absence of said counterselectable marker and in the presence of a selectable marker; and/or

(cb) a further selective medium that allows identification of said host cells only on activation of the readout system;

(d) identifying host cells that contain molecules that:

(da) do not activate said readout system on said at least one selective medium specified in (ca); and

(db) activate said readout system on said selective medium specified in (cb); and

(e) identifying at least one member of said pair or complex of interacting molecules.

2. The method of claim 1, wherein each genetic element carries a counterselectable marker which is different for each genetic element.

3. The method of claim 1 or 2, wherein said pair or complex of interacting molecules is selected from the group consisting of RNA-RNA, RNA-DNA, RNA-protein, DNA-DNA, DNA-protein, protein-protein, protein-peptide, or peptide-peptide interactions.

4. The method of any one of claims 1 or 3, wherein said genetic elements are plasmids artificial chromosomes, viruses or other extra chromosomal elements.

5. The method of any one of claims 1 to 4, wherein said interactions lead to the formation of a functional transcriptional activator comprising a DNA-binding and a transactivating protein domain and which is capable of activating a responsive moiety driving the activation of said readout system.

6. The method of claim 5, wherein said readout system is a detectable protein.

7. The method of claim 6, wherein said detectable protein is encoded from at least one of the genes lacZ, HIS3, URA3, LYS2, sacB or HRPT.

8. The method of any one of claims 1 to 7, wherein said host cells are yeast cells, bacterial cells, mammalian cells, insect cells or plant cells.

9. The method of any one of claims 1 to 8 further comprising transforming or transfecting said host cells with said genetic elements prior to step (a).

10. The method of any one of claims 1 to 9, wherein cell fusion, conjugation or interaction mating is used for the generation of said host cells with said genetic elements prior to step (a).

11. The method of any one of claims 1 to 10, wherein said counterselectable marker selected against in step (ca) is selected from the group of CAN1, CYH2, LYS2, URA3, HPRT and sacB.

12. The method of any one of claims 1 to 11, wherein said selectable marker is an auxotrophic or antibiotic marker.

13. The method of claim 12, wherein said auxotrophic or antibiotic marker is LEU2, TRP1, URA3, ADE2, HIS3, LYS2 or Zeocin.

14. The method of any one of claims 1 to 13, wherein progeny of host cells of step (b) are transferred to storage compartment.

15. The method of claim 14, wherein said transfer is effected or assisted by automation or a picking robot.

16. The method of claim 14 or 15, wherein said storage compartment comprises an anti-freeze agent.

17. The method of any one of claims 4 to 16 wherein said storage compartment is a microtiter plate.

18. The method of claim 17, wherein said microtiter plate comprises 384 wells.

19. The method of any one of claims 1 to 18, wherein said transfer in regular grid pattern in step (c) made or assisted by automation is made by a spotting robot, pipetting or micropipetting device.

20. The method of any one of claims 1 to 19, wherein said regular grid pattern is at densities of 1 to 1000 clones per cm^2.

21. The method of claim 19 or 20, wherein said transfer is made to a planar carrier.

22. The method of any one of claims 19 to 21, wherein said transfer is made by multiple transfers carrying additional host cells to the same position in said regular grid pattern.

23. The method of any one of claims 19 to 22, wherein said planar carrier is a membrane.

24. The method of any one of claims 1 to 23, wherein said identification of said host cells in step (d) is effected by visual means from consideration of the activation state of said readout system.

25. The method of any one of claims 1 to 24, wherein said identification of said host cells in step (d) is effected by digital image storage, analysis or processing.

26. The method of any one of claims 1 to 25, wherein said identification of said at least one member of said pair of interacting molecules is effected by nucleic acid hybridization, antibody binding or nucleic acid sequencing.

27. The method of claim 25, wherein said identification made by said antibody reaction or said hybridization is effected using regular grids of said at least one member or of said genetic information encoding said at least one member.

28. The method of claim 27, wherein construction of said regular grids is effected by automation or a spotting robot.

29. The method of any one of claims 26 to 28, wherein said identification is effected by digital image storage, processing or analysis.

30. The method of any one of claims 26 to 29 wherein nucleic acid molecules, prior to said identification, are amplified by PCR or are amplified in as a part of said genetic elements, preferably in bacteria and most preferably in E.coli.

31. The method of any one of claims 1 to 30, wherein, prior to step (a) a preselection against clones that express a single molecule able to activate the readout system is carried out on culture media comprising a counterselective compound.

32. The method of claim 31, wherein said counterselective compound is 5-fluoro orotic acid, canavanine, cycloheximide or α-amino-adipate.

33. A method for the production of a pharmaceutical composition comprising formulating said at least one member of the interacting molecules identified by the method of any one of claims 1 to 32 in a pharmaceutically acceptable form.

34. A method for the production of a pharmaceutical composition comprising formulating an inhibitor of the interaction of the interacting molecules identified by the method of any one of claims 1 to 32 in a pharmaceutically acceptable form.

35. A method for the production of a pharmaceutical composition comprising identifying a further molecule of a cascade of interacting molecules, of which the at least one member of said interacting molecules identified by the method of any one of claims 1 to 32 is a part of or identifying an inhibitor of said further molecule.

36. Kit comprising at least one of the following:

(i) host cells as identified in any of the preceding claims and at least one genetic element comprising said genetic information specifying at least one of said possibly interacting molecules and

optionally containing a counter-selectable marker as specified in any of the preceding claims;

(ii) host cells as identified in any of the preceding claims and at least one genetic not comprising genetic information specifying at least one of said potential interacting molecules and optionally containing a counter-selectable marker as specified in any of the preceding claims;

(iii) at least one genetic element comprising said genetic information specifying at least one of said potentially interacting molecules and optionally containing a counter-selectable marker as specified in any of the preceding claims;

(iv) at least one genetic element not comprising genetic information specifying at least one of said potentially interacting molecules and optionally containing a counter-seletable marker as specified in any of the preceding claims;

(v) host cells comprising at least one and preferably at least two of said genetic elements specified in (iii) or (iv);

(vi) at least one planar carrier carrying nucleic acid or protein from said host cells comprising at least one member of said genetic elements as specified in any of the preceding claims wherein said nucleic acid or protein is affixed to said carrier in grid form and optionally solutions to effect hybridization or binding of nucleic acid probes or proteins to said molecules affixed to said grid;

(vii) at least one storage compartment, planar carrier or computer disc comprising or/and characterizing genetic elements, host cells, storage compartments or carriers identified in any of (i) to (vi); and/or

(vii) at least one yeast strain comprising a *can*1 and a *cyh*2 mutation.

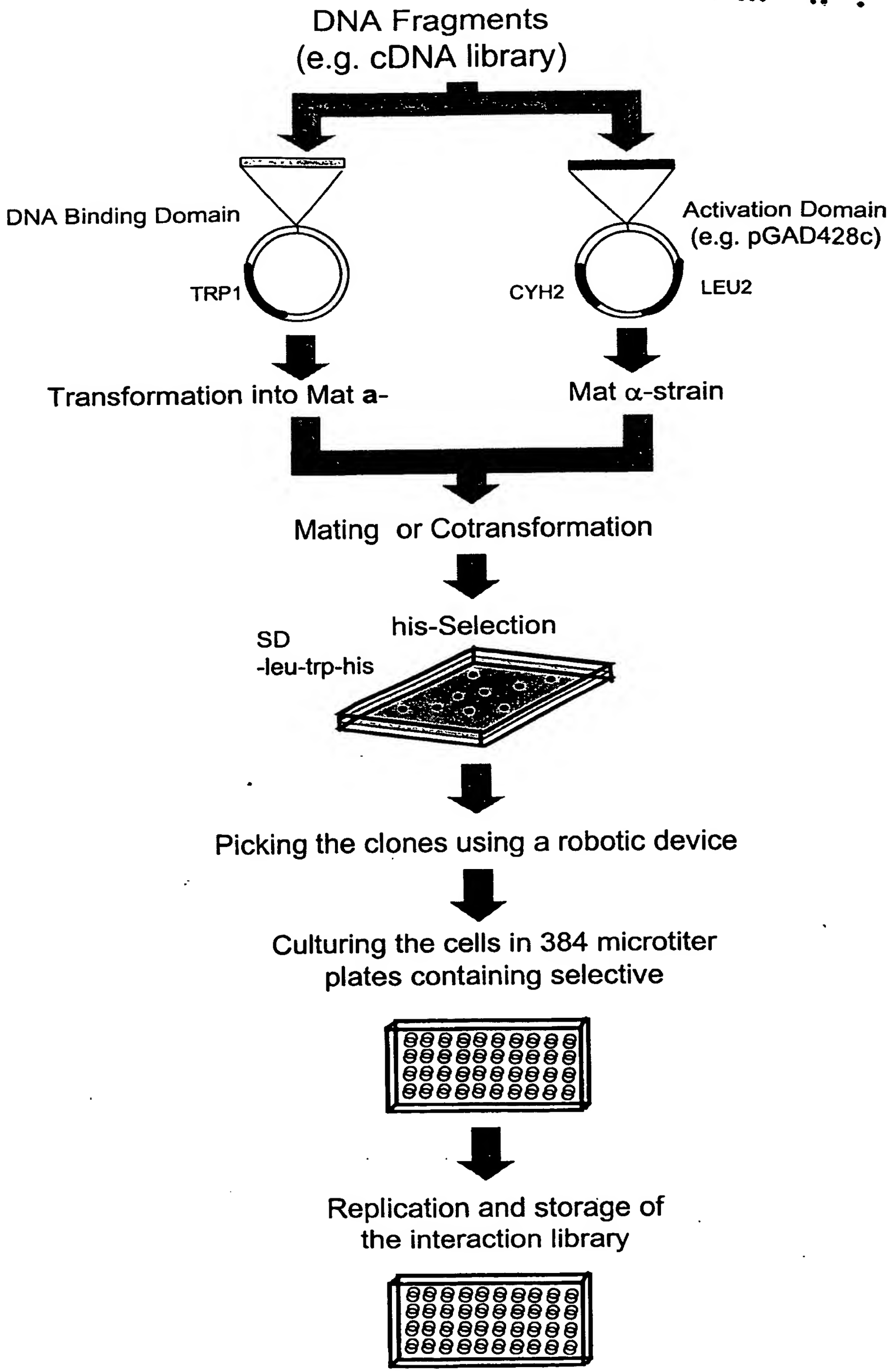
37. The kit of claim 36, wherein said host cells of (i), (ii) or (v) are contained in at least one storage compartment.

38. The kit of claim 36 or 37, wherein said genetic information or said potentially interacting molecules encoded by said genetic information as specified in (i) or (iii) are contained in at least one storage compartment.

ABSTRACT

The present invention relates to an improved method for the identification and optionally the characterization of interacting molecules designed to detect positive clones from the rather large numbers of false positive clones isolated by conventional two-hybrid systems. The method of the invention relies on a novel combination of selection steps used to detect clones that express interacting molecules from false positive clones. The present invention further relates to a kit useful for carrying out the method of the invention. The present invention provides for parallel, high-throughput or automated interaction screens for the reliable identification of interacting molecules.

Figure 1



Spotting of yeast cells onto a nylon membrane
Transfer of these membranes to different selective media

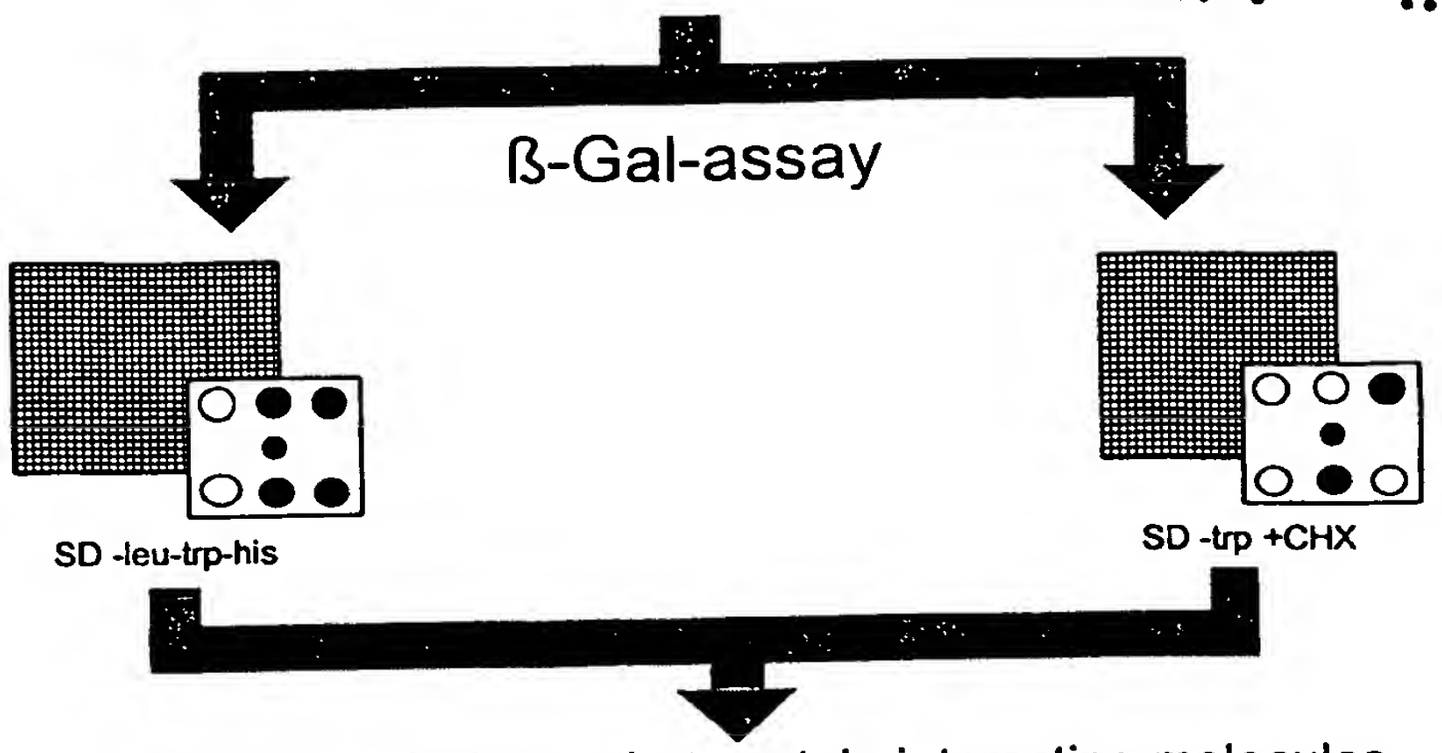


Figure 1 continued

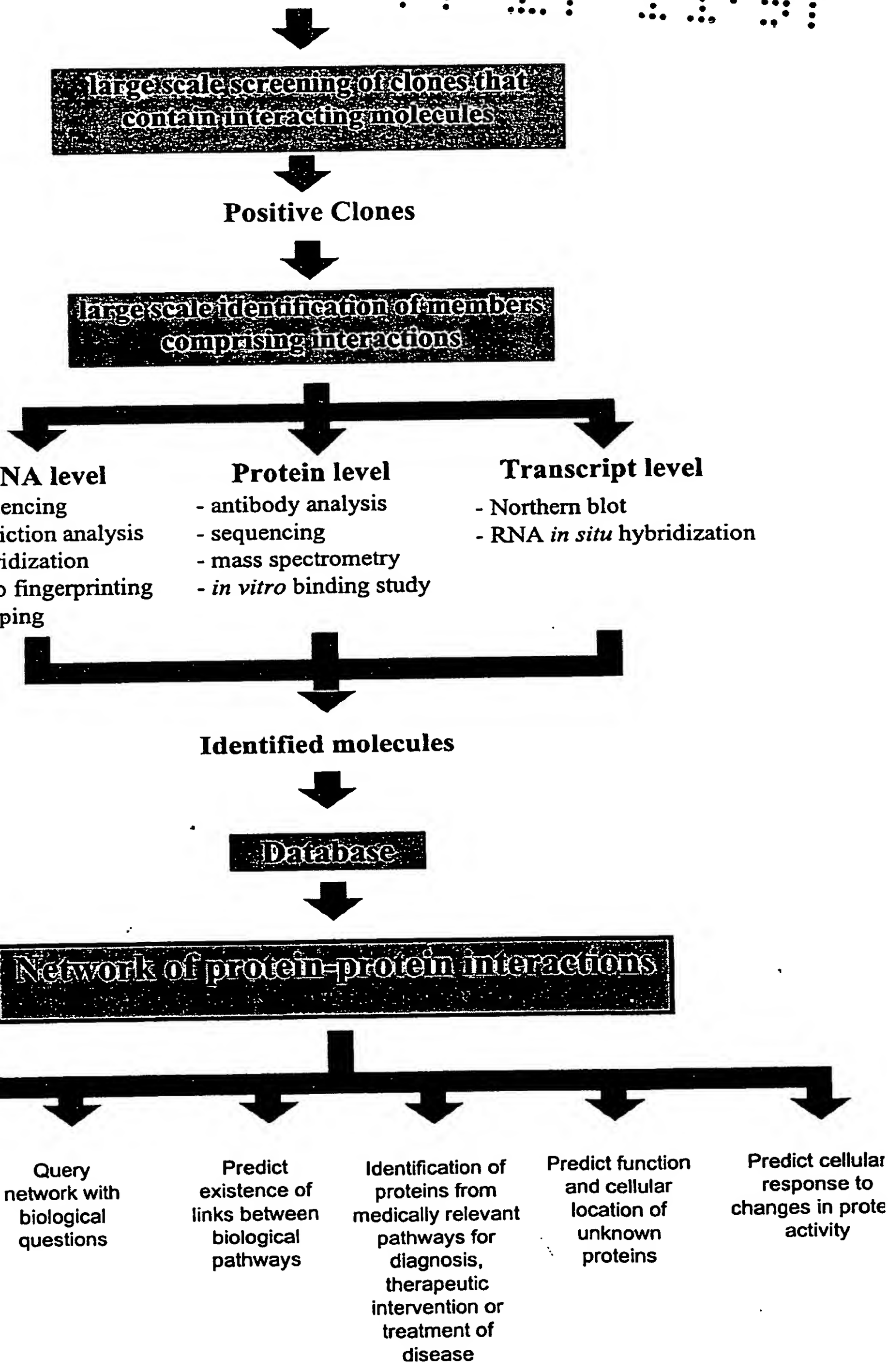
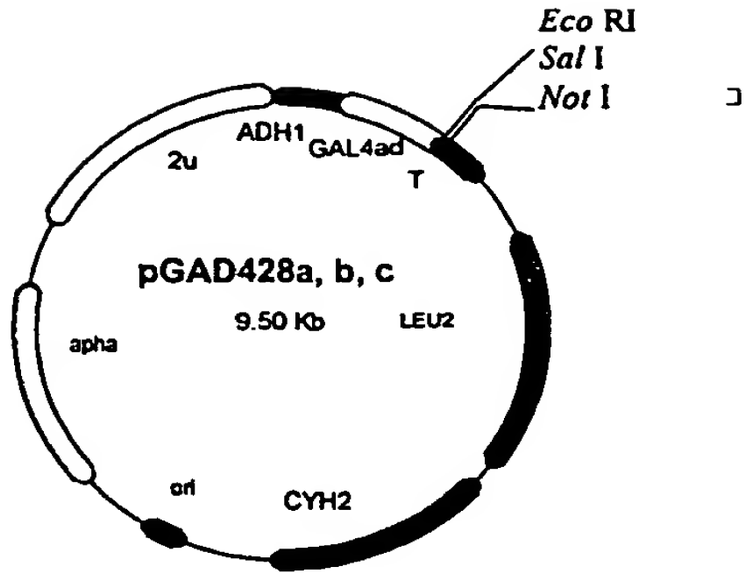


Figure 2



a) TCG AGT CGA CGC GGC CGC TAA CCG G
Sal I *Not* I STOP

b) TCG AGG TCG ACG CGG CCG CAG TAA CCG G
Sal I *Not* I STOP

c) TCG AGA GTC GAC GCG GCC GCT TAA CCG G
Sal I *Not* I STOP

Fig. 3a and b

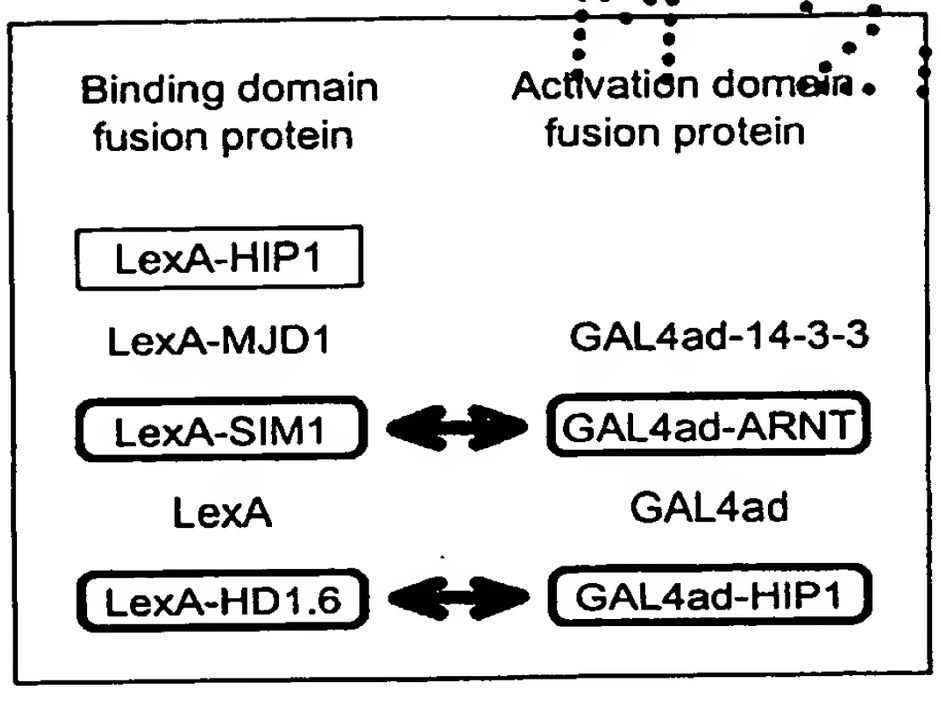
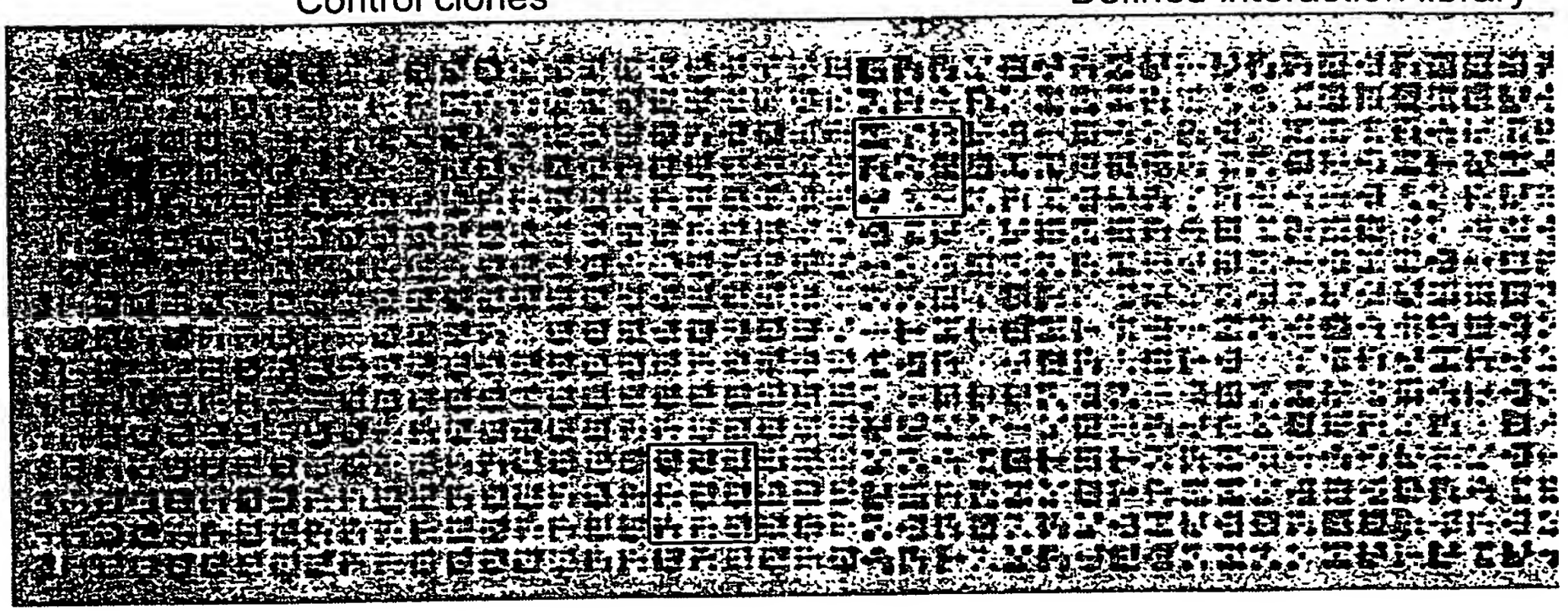

Binding domain fusion protein
Activation domain fusion protein
LexA-HIP1
LexA-MJD1
GAL4ad-14-3-3
LexA-SIM1
GAL4ad-ARNT
LexA
GAL4ad
LexA-HD1.6
GAL4ad-HIP1

Figure 4

a. SD-leu-trp-his



b. SD-trp+CHX

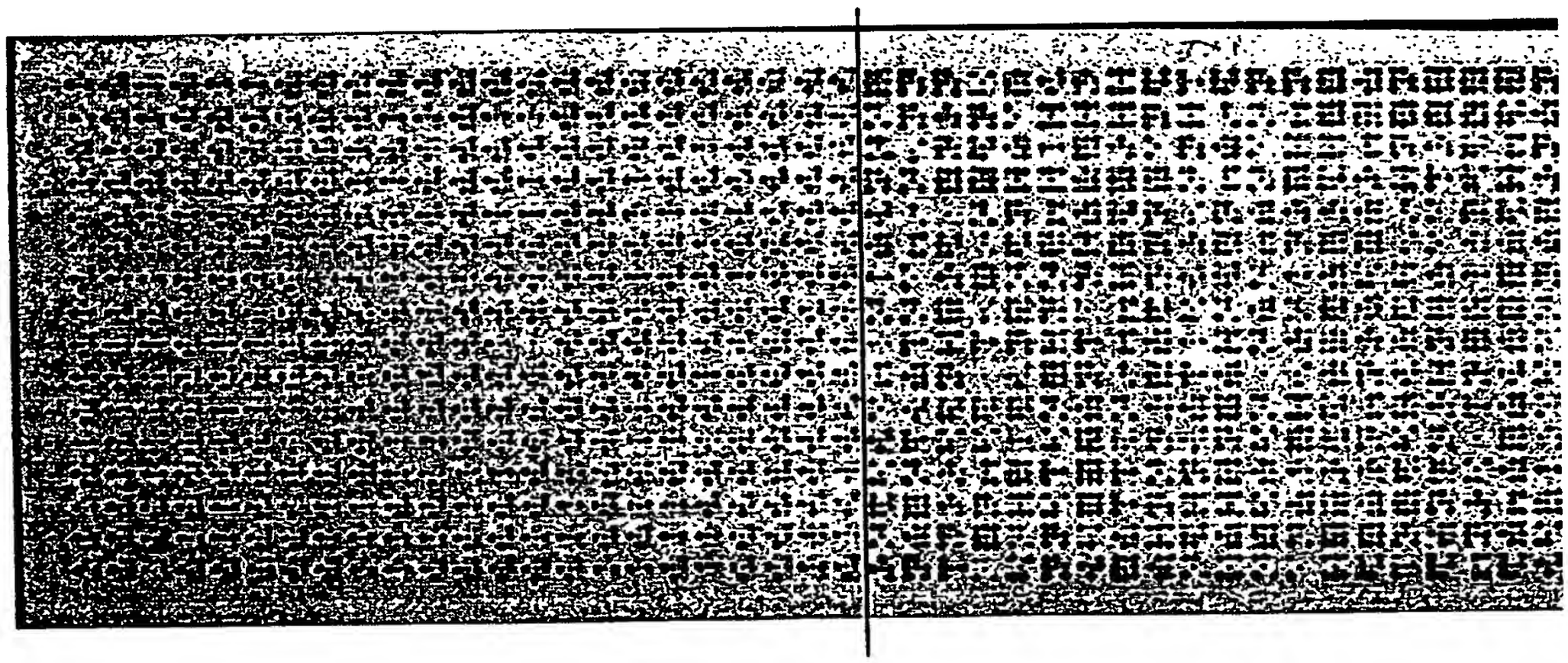
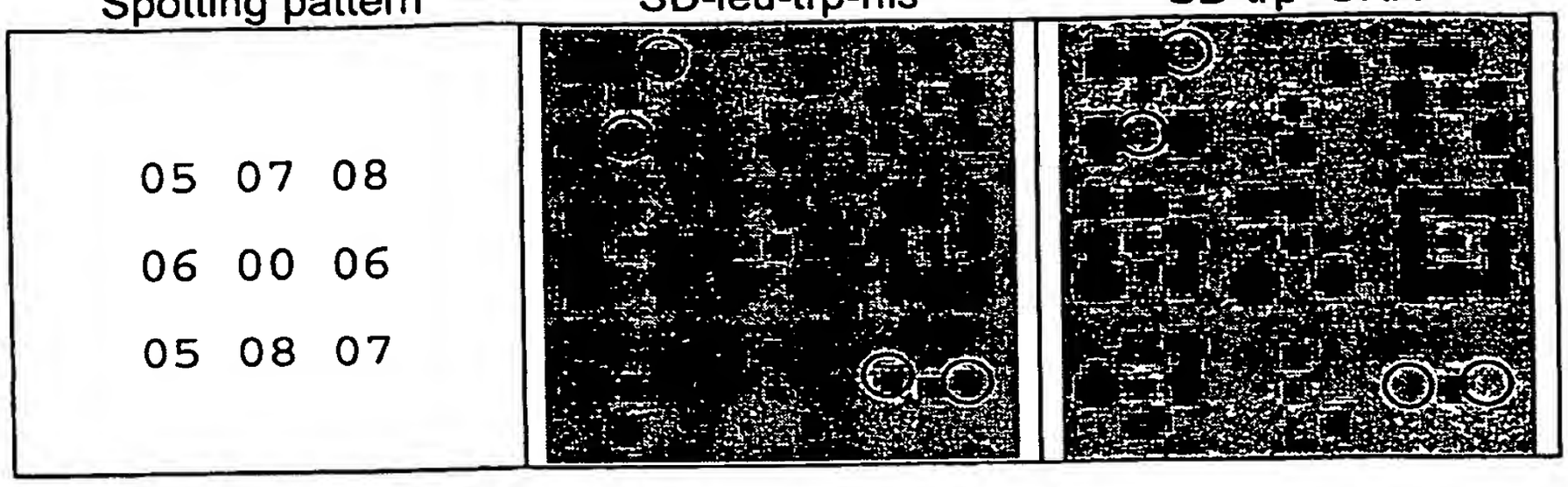


Figure 5

a.



b.

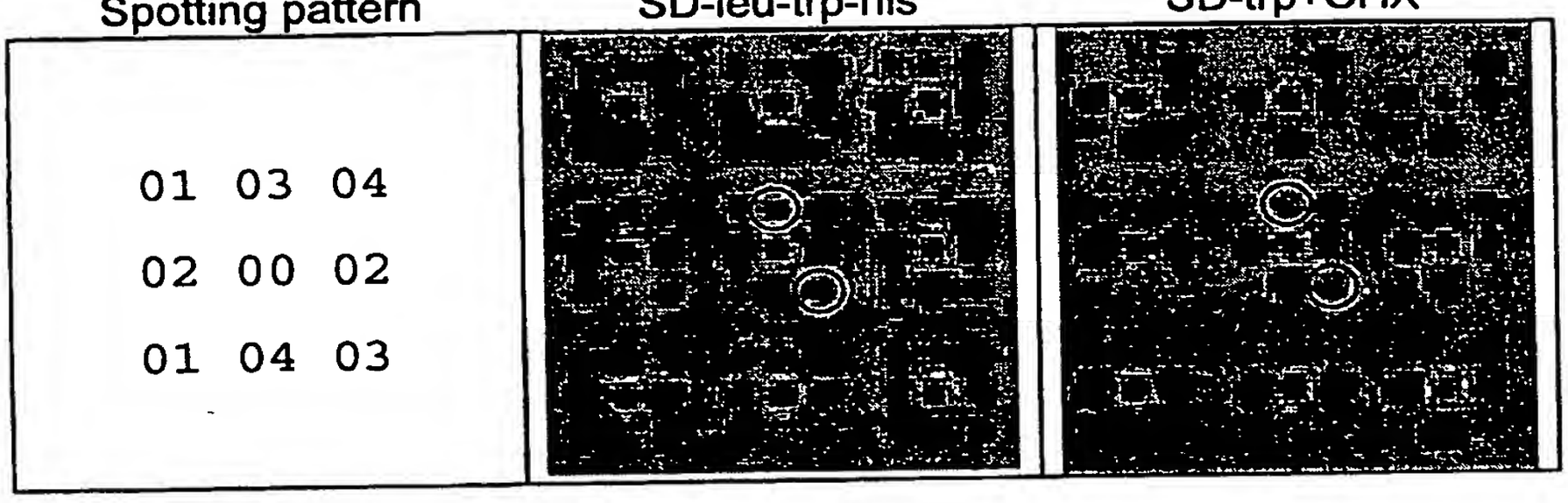


Figure 6

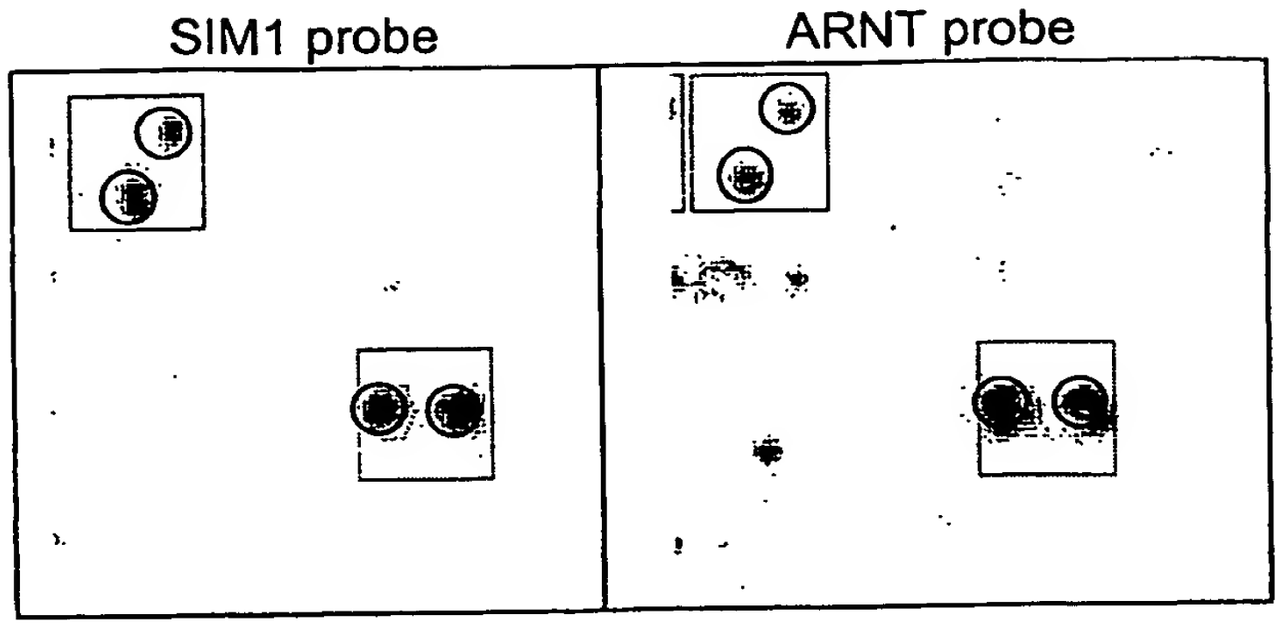


Figure 7

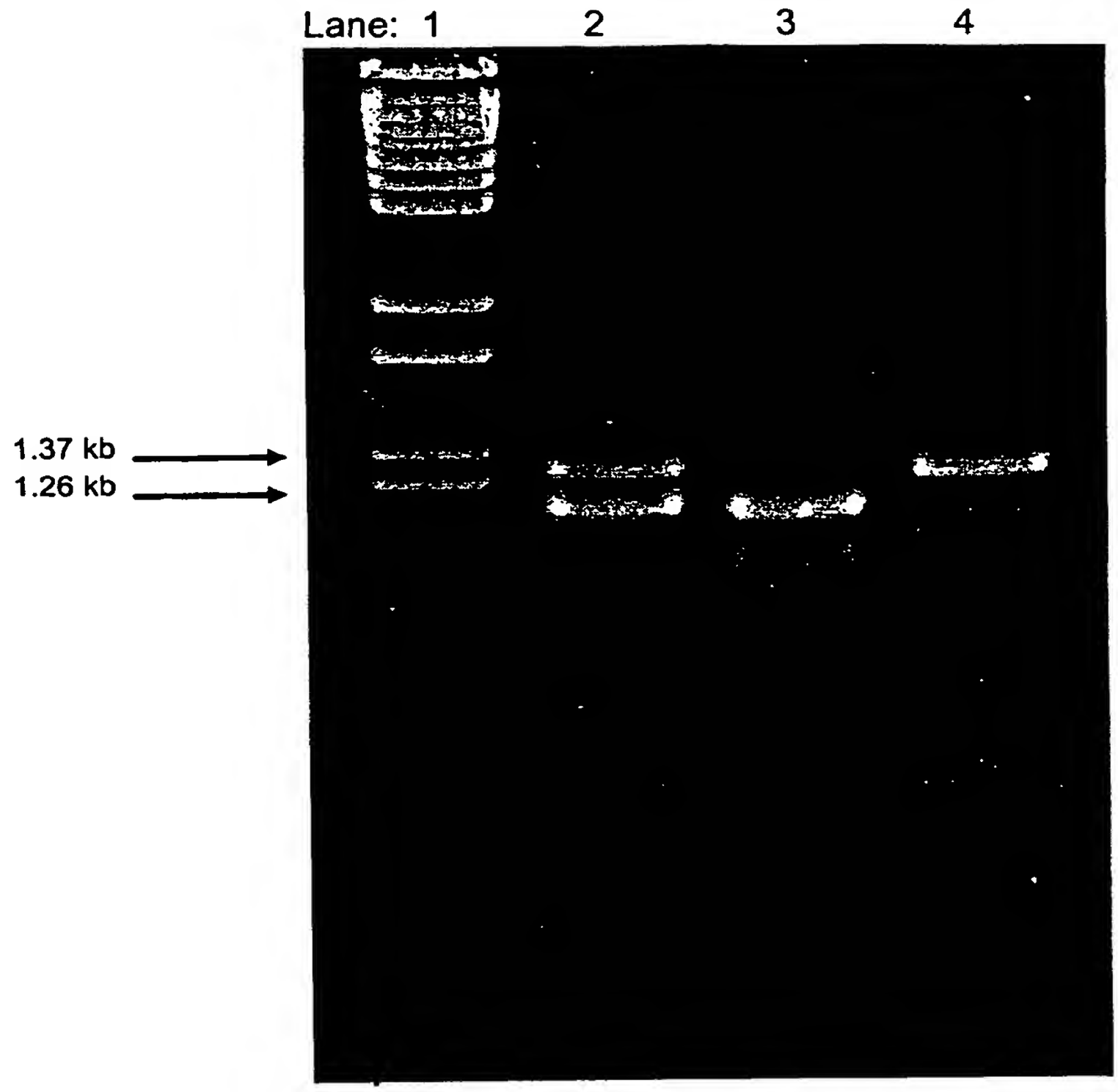


Figure 8